Prospective Detection of Preclinical Lung Cancer: Results from Two Studies of Heterogeneous Nuclear Ribonucleoprotein A2/B1 Overexpression[1]

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ABSTRACT

The United States lung cancer epidemic has not yet been controlled by present prevention and treatment strategies. Overexpression of a M_r 31,000 protein, heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1, had shown promise as a marker of lung cancer. In a pilot study of archived preneoplastic sputum specimens, hnRNP A2/B1 overexpression more accurately detected preclinical lung cancer than standard cytomorphology. In separate, ongoing prospective studies, sputum is collected annually from stage I resected non-small cell lung cancer patients at high risk of developing a second primary lung cancer and Yunnan tin miners at high risk of primary lung cancer. After the first year of follow-up, preclinical detection of lung cancer by routine cytology was compared with hnRNP A2/B1 overexpression as measured by quantitative densitometry of immunostained slides. Up-regulation of hnRNP A2/B1 in sputum specimens accurately predicted the outcome in 32 of 40 primary lung cancer and control patients within 12 months, whereas cytological change suggestive of lung cancer was found in only 1 patient. In the primary lung cancer study, overexpressed hnRNP A2/B1 accurately predicted the outcome in 69 of 94 primary lung cancer and control miners, whereas only 10 with primary lung cancer were diagnosed cytologically. These two prospective studies accurately predicted that 67 and 69% of those with hnRNP A2/B1 up-regulation in their sputum would develop lung cancer in the first year of follow-up, compared with background lung cancer risks of 2.2 and 0.9% (35- and 76-fold increase, respectively). Using sputum cells to monitor hnRNP A2/B1 expression may greatly improve the accuracy of preclinical lung cancer detection.

INTRODUCTION

Because more than 80% of lung cancer cases result from cigarette smoking, primary prevention has been the focus of cancer control efforts (1–4). However, although the prevalence of cigarette smoking in the United States declined to 25% in 1993 (5), the age-adjusted mortality from lung cancer has not yet shown a similar decline (6), in part because of the persisting lung cancer risk of former smokers (7–9). The risk among the estimated 46 million current and 46 million former smokers led to 159,000 lung cancer deaths in the United States in 1996. With 66,000 (41%) deaths in women, lung cancer is now the commonest cause of cancer mortality among both sexes (5).

Twenty-five % of the United States adult population are former smokers. The interplay of their persisting risk and advancing age may delay any improvement in lung cancer mortality over the next 20 or 30 years, despite successful efforts at smoking prevention. Due to persistent, widespread bronchial epithelial cell injury, former smokers maintain an elevated risk for lung cancer. For the first time, major thoracic oncology centers have diagnosed more new cases of lung cancer in former smokers rather than current smokers (10). Moreover, the ther-

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apeutic approach used to combat clinically apparent lung cancer often controls local disease but results in a survival rate of only 13% because of the failure to control metastases (11). Some 40% of these deaths will be among patients in their most productive years, and each smoking-related death results in an average loss of 15 years of expected life (4). Furthermore, cell type and stage-specific costs of lung cancer management have recently been estimated for Canada (12). Allowing for the fact that the annual number of United States lung cancer patients is 10 times greater, a conservative translation of these estimates would lead to costs of $4.2 billion a year for the United States. Better understanding of lung cancer biology and new approaches to early therapy now call for a fresh look at preclinical detection.

In earlier reports, we identified potentially useful lung cancer biomarkers from their expression by nonneoplastic sputum cells archived during the collaborative National Cancer Institute Early Lung Cancer screening trial and later by tumor from these individuals who developed lung cancer. In these archived sputum and tumor specimens, we have identified gene products (tumor-associated and differentiation protein antigens in 20 of 22, 91%, who later developed cancer; Ref. 13), mutations of K-*ras* and *p53* (in 8 of 15, 53%; Ref. 14), and microsatellite alterations (in 7 of 35, 20%; Refs. 1 and 15) as potential markers of subsequent malignancy.

Comparing test agreement with the actual state of the patient (determined from archived specimens), the most accurate [(true positive + true negative)/total = 88%] of these markers was the up-regulated antigen detected by monoclonal antibody 703D4 (13). We reported recently the antigen target of 703D4 to be hnRNP[5] A2/B1 (16). This family of RNA-binding proteins is responsible for the posttranscriptional regulation of gene expression by capping, splicing, polyadenylation, and cytoplasmic transportation of mRNAs (17) and appears to be dysregulated in malignant cells (16). Despite the promise of such new biological approaches, sputum cytomorphology and chest radiography are the only lung cancer screening techniques to have undergone prospective evaluation.

We now provide an advance report of two prospective studies that screen exfoliated airway cells of high-risk individuals for an accurate marker of preclinical lung cancer. Overall, each study's goal is to compare the accuracy of hnRNP A2/B1 overexpression by exfoliated sputum epithelial cells with routine sputum morphology. These studies were initiated to address two specific questions: (*a*) does hnRNP A2/B1 overexpression correctly detect preclinical lung cancer; and (*b*) can it do so in the absence of dysplastic morphological changes in sputum epithelial cells. An 11-center collaborative study was conducted by the LCEDWG (1, 18) in stage I resected, disease-free patients whose annual risk of SPLC is between 1 and 5% (19). In a second study, hnRNP A2/B1 expression was evaluated in YTC miners, a Chinese industrial population with extensive exposures to tobacco smoke, radon, and arsenic, whose average annual incidence of primary lung cancer (1°LC) is 1% (20). These descriptive studies continue as blinded, prospective, observational designs. No treatment decisions result from hnRNP A2/B1 assay. Initial screening and first-year follow-up data are presented separately for each study. Because of the magnitude and consistency with findings observed previously in archived material and the potential biological and clinical importance of these results, we feel that a report at this time is warranted.

MATERIALS AND METHODS

SPLC Population and Study Design

Investigators at institutions formerly participating in the National Cancer Institute's Lung Cancer Study Group (21–24), plus other institutions with active surgical oncology programs, have formed the collaborative LCEDWG. Study patients were identified by these investigators after complete resection of NSCLC. Patients were eligible regardless of age, gender, ethnic background, Karnofsky score, or smoking status. Tumor-Node-Metastasis staging was based on the extent of the cancer at resection (25), and cell type was classified according to WHO diagnostic criteria (26). Provided a patient underwent biopsy of at least one mediastinal node and all biopsied mediastinal nodes were negative, anyone with T_1N_0 or T_2N_0 disease who had not developed either recurrence or SPLC 6 weeks or more after surgical resection was eligible. If node sampling was not done, 2 years must have elapsed since surgery with no known or suspected metastases beyond the mediastinum.

Before enrolling patients, each LCEDWG investigator received local Institutional Review Board approval, established a sputum induction facility, and provided staff to receive specimen collection training and approval during a site visit by a cytotechnologist from the Johns Hopkins University School of Hygiene. Techniques for specimen production and handling were as follows. To enhance specimen quality, each patient performed a 15-min hypertonic saline induction. Fresh sputum was smeared on glass slides for Papanicolaou staining, and the remaining sputum was homogenized, concentrated, and placed in Saccomanno's preservative (2% polyethylene glycol 1450 in 50% ethanol; Ref. 27). On arising over the following 3 days, the patient collected postinduction sputum in Saccomanno's preservative, then mailed in the pooled specimen. If routine cytological examination at the receiving institution showed the presence of neoplastic cells, the patient underwent evaluation for SPLC (or recurrence) by the treating physician. All lung tumors were histologically confirmed. SPLC was defined as lung cancer that had to be a different histological cell type than the primary, if it appeared within 2 years of primary resection. If SPLC appeared more than 2 years after resection, it could be of the same cell type, provided that it had the characteristics of a primary cancer and arose in a different lobe (19). All screening specimens were sent to Johns Hopkins for analysis.

Chinese Population and Study Design

Active and retired Chinese tin miners could volunteer for annual primary lung cancer screening if they were older than 40, had worked underground for more than 10 years, had no previous malignancy (except nonmelanoma skin cancer), and gave

[5] The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; LCEDWG, Lung Cancer Early Detection Working Group; YTC, Yunnan Tin Corp.; SPLC, second primary lung cancer; NSCLC, non-small cell lung cancer; JHLP, Johns Hopkins Lung Project.

informed consent. At registration, standardized interviews recorded age, gender, and ethnic background and smoking, occupational, and nutritional histories. Each annual sputum specimen produced during a hypertonic saline induction was examined, and each miner underwent annual chest radiography. The miners in whom lung cancer was detected were advised to undergo a diagnostic work-up at the YTC Workers' General Hospital in Geiju City. The criteria used for cell type and staging were similar to those described earlier for the SPLC. Analysis of specimens was based on a prospective, case-cohort design. At the conclusion of the first year of follow-up, baseline screening sputum specimens of miners who developed primary lung cancer were sent to Johns Hopkins for analysis. For comparison, a randomly selected subcohort of controls, age-stratified by the expected distribution of lung cancer cases, was identified at enrollment and then merely followed. At the conclusion of the first year of follow-up, baseline screening sputum specimens of the age-matched subcohort were similarly sent for analysis.

Central Labs/Lab Procedures

Sputum Morphology. The specimen collection, preparation, staining, and quantitation methods used were described during previous evaluations of JHLP archived specimens (13) and were similar for both studies. A single cytopathologist (Y. S. E.) reviewed all slides that showed even moderate atypical metaplasia, as well as a sample of the negatives. The presence of "regular" metaplasia was considered normal. Epithelial cells with regular metaplasia were visually selected for analysis by a cytotechnologist who had no knowledge of the patients' clinical status. Two slides per patient were scanned for 5–10 characteristic fields. Each field contained at least one regularly metaplastic epithelial cell, and a minimum of five such cells were evaluated for each patient.

Immunocytochemistry and Cell Culture Controls. A single lot of monoclonal antibody to hnRNP A2/B1 (designated 703D4) was purified from mouse ascites using a protein A column and discontinuous glycine NaCl/citrate gradient (Pierce, Rockford, IL; Ref. 28). This purified antibody (10 μg/ml) was applied to cytospin slides (Shandon, Pittsburgh, PA) of each patient's specimen and positive control slides. For negative controls, the primary antibody was replaced by a similar protein concentration of mouse IgG_{2b} nonimmune serum. Immunostaining consistency was achieved by applying Vectastain Elite ABC kit reagents (Vector Laboratories, Burlingame, CA) with a semiautomated capillary-gap technique (Biotek Instruments, Chicago, IL) following Gupta's method (29). Slides were interpreted by a study immunocytopathologist (P. K. G. or W. H. Z.; Fig. 1) before automated measurement. Control slides were made from American Type Culture Collection human bronchogenic cancer cell lines HTB58 (squamous cell cancer) and Calu-3 (adenocarcinoma) mixed with normal sputum, placed in Saccomanno's preservative, and processed as above.

Image Cytometry

Koehler illumination was established on a Zeiss Axiomat microscope (Carl Zeiss, Oberkochen, Germany). The dynamic range of the high resolution video camera (Hamamatsu Photonic Systems, Japan) was established using the positive control, then

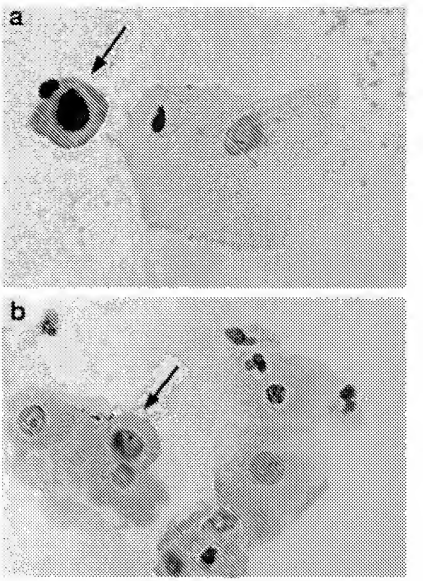

Fig. 1 Images of sputum epithelial cells showing mild atypical metaplasia and expressing hnRNP A2/B1 as detected by monoclonal antibody 703D4 and stained with diaminobenzidine and hematoxylin (×2000). *a*, hnRNP A2/B1 overexpression in an epithelial cell (*arrow*) from a sputum specimen preceding a SPLC. *b*, an epithelial cell (*arrow*) expressing a normal (background) level of hnRNP A2/B1 in a specimen from a patient who remained normal. A nearby alveolar macrophage (MΦ) is laden with carbon particles.

light transmission was standardized with absorbance (*A*), 0.2 and *A*, 0.4 neutral density filters. To optimize the transmitted light frequency for the brown diaminobenzidine that labels hnRNP A2/B1 expression and the blue (hematoxylin) counterstain, Omega narrow-band filters of 600 nm (range, 590–610 nm) and 510 nm (range, 500–520 nm), respectively, were used (30). Transmission was recorded by a digital image processor (Metamorph version 2.0; Universal Imaging, West Chester, PA). Background-subtracted, shading-corrected images of each immunostained field at both wavelengths were then saved to an optical drive (Panasonic/Matsushita Co., Osaka, Japan). Interpretations of Papanicolaou stained and immunostained slides and optical/electronic quantitation were entered into the database maintained by the Johns Hopkins Oncology Biostatistics Coordinating Center. Finally, all slides and an aliquot of each specimen were placed in storage.

Statistical Considerations

The primary statistical end point for these studies is to estimate the accuracy of positive immunostaining (compared to

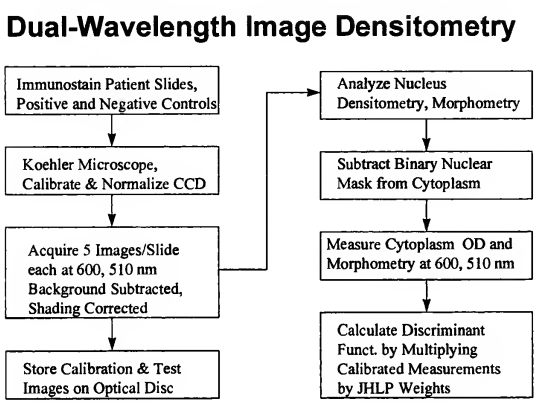

Fig. 2 Previously validated dual-wavelength image densitometry procedure algorithm (31).

routine morphological sputum surveillance) for detection of subsequent lung cancer: a SPLC (recurrences were removed from the denominator and not counted) in the SPLC population; and a primary lung cancer in the YTC population. Using the proportional hazards regression model, each of these studies will have 90% power to recognize detection rates of double (hazard ratio, 2.0) or greater for immunostaining if 90 lung cancers are observed (two-sided $\alpha = 0.05$). This power calculation assumes that approximately 50% of lung cancers are in immunostain-positive patients. If only a small proportion of lung cancers are in immunostain-positive patients, a larger sample size will be required. For example, if only 20% of lung cancers are in stain-positive patients, 130 lung cancers will have to be observed. These studies have been designed to evaluate the data after the first year to more accurately estimate the proportion of patients who stain positively. The required sample size will be adjusted based on these initial data. Both the magnitude of these differences and their significance may, of course, change by the end of these studies, which now continue to their designed accrual. χ^2 and t tests were used to assess the significance of frequency and mean differences between those with and without cancer.

At the end of the first year, sputum morphology, immunocytochemistry, and clinical cancer/noncancer end point status was evaluated for SPLC patients whose follow-up had ended (developed cancer, died from other causes, or withdrew). Similar sputum marker and clinical end points were determined for YTC participants who developed cancer plus age-matched controls. YTC controls were selected from a random 10% sample identified at initial screening as a "control" subcohort. If individuals from this control subcohort developed cancer, they became "cases" and were replaced.

Results of quantitative image cytometry were interpreted according to an algorithm (Fig. 2) and a discriminant function (SPSS-Win version 6.0; SPSS Inc., Chicago, IL) developed previously from JHLP sample data (31). The discriminant function incorporated a similar clinical end point (cancer/no cancer) grouping variable and two independent variables based on the optical densities at 600 and 510 nm. Prior probabilities for each (case/control) group were assumed to be equal. This algorithm was applied to the test specimens from the SPLC and primary lung cancer studies to predict clinical outcome. Absorbance measurements of epithelial cells from each specimen were averaged over each wavelength and used to classify specimens as neoplastic on the basis of a linear discriminant function (D):

$$D = \beta_0 + \beta_1 (\text{Av. absorbance}_{600})^{1/2} - \beta_2 (\text{Av. absorbance}_{510})^{1/2}$$

The cutpoint value of D (indicating neoplasia) and the weights β_0, β_1, and β_2 were determined in advance from reference sputum specimens of JHLP participants who developed NSCLC, small cell lung cancer, or no lung cancer at all (13). The sensitivity and specificity of the prospective discriminant score classification and their exact 95% binomial confidence limits were then calculated.

RESULTS

SPLC Detection. Accrual of patients with resected stage I NSCLC was begun in January 1992 with a 3-year goal of 1000 patients. After 41 months, 660 patients (638 eligible, 2/3 of the goal) have been registered. Among the 595 patients with satisfactory specimens on first examination, we recognized 13 SPLCs and 16 recurrent lung cancers consistent with 13 of each expected during the first year on the basis of 435 person-years of follow-up. Another 27 patients have died from other causes or withdrawn from the study, for an overall total of 56 for whom follow-up has been completed (Table 1). When entry characteristics were compared between those with follow-up completed and the 539 who remain in active follow-up at the end of the first year, no significant differences were identified. The SPLC population was primarily white and nearly 60% were men (Table 1). Although 90% of the patients had smoked in the past,

Table 1 Entry characteristics of 595 subjects at risk of SPLC by outcome group

	Active follow-up (n = 539)		Completed follow-up								
			Second primary (n = 13)			Recurrence (n = 16)			Noncancer (n = 27)		
	%	Mean (range)	%	Mean (range)	P^a	%	Mean (range)	P	%	Mean (range)	P
Race[b]					0.232			0.185			0.412
White	90.1		100.0			100.0			85.2		
Nonwhite	9.9		0.0			0.0			14.8		
Gender[c]					0.059			0.237			0.758
Male	58.6		84.6			43.8			55.6		
Female	41.4		15.4			56.2			44.4		
Age at enrollment (yr)[c]		66.0 (33–89)		68.4 (61–79)	0.142		63.3 (53–79)	0.234		68.3 (40–83)	0.227
Smoking status[c]					0.149			0.909			0.135
Current	14.1		30.7			13.3			22.2		
Former	76.0		69.2			80.0			59.3		
Never	9.9		0.0			6.7			18.5		
Age at diagnosis (yr)		62.3 (32–85)		65.4 (53–79)	0.260		60.7 (53–71)	0.489		64.9 (39–79)	0.189
Karnofsky score		95.4 (50–100)		92.3 (70–100)	0.222		97.5 (60–100)	0.363		94.8 (60–100)	0.739
Cell type of primary[d]					0.108			0.530			0.913
Squamous	33.7		23.1			25.0			29.6		
Large cell	6.9		15.4			0.0			11.1		
Adenocarcinoma	43.3		38.5			62.5			40.7		
Bronchoalveolar	11.9		7.7			6.3			14.8		
Mixed	2.7		15.3			16.3			3.7		
Other	1.5		0.0			0.0			0.0		
Cell type, second primary											
Squamous			15.4								
Large cell			15.4								
Adenocarcinoma			30.8								
Mixed			15.4								
Other			15.4								
Missing			7.7								

[a] P test differences between subgroups with completed follow-up and those in active follow-up.
[b] There are 15 cases whose race was unknown.
[c] There are 13 cases whose gender, age at enrollment, or smoking status was unknown.
[d] There are 17 cases whose primary cell type was unknown.

three-fourths of them considered themselves former smokers at registration (no longer a regular cigarette smoker). Their mean age at enrollment, 3.6 years after primary resection, was 66.5 years. Good health was reflected by their average Karnofsky score (95.2). The most commonly resected cell type for the primary tumor was adenocarcinoma (43.8%), and when combined with the bronchoalveolar subtype (11.5%), adenocarcinoma constituted 55.3% of the resected primary tumors. The distribution of primary cell types was not different among those who developed a second primary, a recurrence, or remained cancer-free. Adenocarcinoma also was the most common SPLC (4 of 13, 31%). Squamous cell, mixed adenosquamous, large cell, and small cell each accounted for 2 of 13 patients (15%), whereas one SPLC patient died before histological confirmation.

Cytological review of 582 of 595 (98%) available initial sputum specimens showed that 68.3% contained only normal morphology, 13.8% showed slight atypical metaplasia, 1.1% exhibited moderate atypical metaplasia, and one case (0.1%) showed grave atypical metaplasia. None of the specimens showed neoplastic morphology, and there was no significant association between the extent of cytological abnormality and the cell type of the primary tumor. Immunostaining intensity of regularly metaplastic cells was evaluated for each individual. The results of immunostaining among all 595 SPLC patients are presented in Table 2. Compared with those who did not have cancer (A, 0.372, Table 2) or those in active follow-up who have not yet reached an end point (A, 0.369), sputum cells of persons who later developed SPLC overexpressed hnRNP A2/B1, as indicated by a significantly greater absorbance at 600 nm (A, 0.445). Sputum cells from those whose lung cancer recurred stained with an intermediate absorbance (A, 0.410). Sputum epithelial cells of current smokers stain with a greater density (A, 0.409) than do cells of former or never smokers (A, 0.370 and 0.330, respectively). We observed an intraindividual variance in staining intensity of A, 0.034.

Overall, the risk of developing SPLC during the first year was 13 of 595 (2.2%; Table 3). Of the patients who overexpressed hnRNP A2/B1, 10 of 15 (67% positive predictive value) developed SPLC 10–12 months after their initial examination. Only 3 of the 25 predicted to be negative (12%) developed SPLC (relative risk, 5.6; sensitivity, 77%; specificity, 82%) for an overall accuracy of 80%. Evaluation of the sputum of the 13 SPLCs for morphological criteria detected only 1 patient with preclinical evidence suggesting neoplasia (grave atypical metaplasia; sensitivity, 8%). These data indicate that immunostaining for hnRNP A2/B1 overexpression increased the sensitivity of

Table 2 Distribution of absorbances[a] at 600 nm by study, race, gender, age group, smoking, and end point status

	Absorbance at 600 λ		
	Mean ± SD	Range	*P*
Characteristics of 595 subjects at risk for SPLC			
Race			0.2314
White	0.373 ± 0.100	0.048–0.756	
Nonwhite	0.356 ± 0.088	0.175–0.581	
Gender			0.2694
Male	0.375 ± 0.100	0.129–0.756	
Female	0.366 ± 0.097	0.048–0.668	
Age at enrollment			0.5459
≤60	0.380 ± 0.110	0.048–0.756	
61–65	0.368 ± 0.093	0.154–0.696	
66–70	0.369 ± 0.100	0.129–0.630	
>70	0.367 ± 0.091	0.101–0.659	
Smoking status			0.0001
Current	0.409 ± 0.111	0.129–0.756	
Former	0.370 ± 0.096	0.101–0.696	
Never	0.330 ± 0.085	0.048–0.513	
End point status			0.0177
SPLC	0.445 ± 0.084[b]	0.332–0.622	
Recurrent lung cancer	0.410 ± 0.090	0.275–0.518	
Noncancer	0.372 ± 0.078	0.248–0.600	
Nonendpoint	0.369 ± 0.100	0.048–0.756	
Characteristics of 94 subjects at risk for primary lung cancer[c]			
Age at enrollment			0.5286
≤60	0.403 ± 0.200	0.128–0.854	
61–65	0.407 ± 0.185	0.142–0.848	
66–70	0.486 ± 0.192	0.175–0.880	
>70	0.426 ± 0.231	0.119–0.796	
Smoking status			0.8475
Current	0.428 ± 0.202	0.119–0.880	
Former	0.421 ± 0.189	0.142–0.843	
Never	0.381 ± 0.234	0.128–0.792	
End point status			0.0001
Case	0.543 ± 0.180	0.160–0.880	
Control	0.312 ± 0.145	0.119–0.796	

[a] In biological tissues, absorbances cover a theoretical range from 0.0 (clear) to 1.2 (unable to transmit light). For these samples, absorbance can be roughly considered to be the proportion of background light blocked by hnRNP immunostaining.

[b] Subjects with SPLC have a significantly greater absorbance than either noncancer subjects ($P < 0.05$) or nonendpoint subjects ($P < 0.05$).

[c] All 94 primary lung cancer subjects are Chinese males.

Table 3 Immunodetection of preclinical SPLC by hnRNP overexpression

	Actual group	
Predicted group (test result)	Cancer	No cancer
Cancer (positive) $n = 15$	10 (76.9)	5 (18.5)
No cancer (negative) $n = 25$	3 (23.1)	22 (81.5)
Total $n = 40$	13	27

Overall SPLC risk: 13/595, 2.2%.
Positive predictive value: 10/15, 67%.
Risk among predicted negative: 3/25, 12%.
Relative risk of a positive test: 250/45, 5.6.
Sensitivity: 77%. Exact 95% binomial confidence interval, 46–95%.
Specificity: 82%. Exact 95% binomial confidence interval, 62–94%.

routine sputum cell morphology in detecting SPLC 9-fold (from 8 to 77%).

Primary Lung Cancer Detection. All of the 6285 eligible YTC miners enrolled for screening were Chinese males. Overall, the risk of developing primary lung cancer during the first year was 57 of 6285 (0.9%). All primary lung cancer patients were confirmed with a consensus "best information" diagnosis by a panel of clinicians from YTC and Johns Hopkins. The cell type of the most commonly resected primary tumors was squamous cell carcinoma (48.9%), whereas adenocarcinoma constituted 4.2% of the primary tumors, and large cell and small cell cancers accounted for one case each (2.1%). The remaining 42% chose alternative care without a histological diagnosis.

Investigators at Johns Hopkins who were unaware of the case/noncase status evaluated the sputum specimens for the 57 primary lung cancer patients and 76 age-matched, subcohort controls. Specimens were considered satisfactory for 94 miners with a mean age at enrollment of 63 years (45 patients and 49 controls; Table 4). In 18 of the 39 unsatisfactory specimens, the absence of alveolar macrophages left uncertain whether the sputum specimen sampled the airway below the glottis. The remaining 21 were unsatisfactory for a variety of reasons, including inflammatory cell confounding, hypocellularity, and unsatisfactory staining technique. Although more than 90% had smoked in the past, only two-thirds smoked when they entered the study. Similar proportions of patients who developed primary lung cancer and controls expressed moderate atypia in their sputum (4 of 45 and 4 of 49, respectively, or 9 and 8%). Ten of 45 (22%) of the cancer patients showed neoplastic cells in their sputum, whereas none of the controls did. Compared with controls, patients who developed lung cancer during this study showed hnRNP A2/B1 overexpression as evidenced by significantly greater absorbances of sputum epithelial cells (*A*, 0.543 and 0.312, respectively; Table 2). Similar to the sputum cells of the SPLC patients, those of currently smoking YTC miners stained with greater intensity (*A*, 0.428; *P*, not significant).

Of the 54 predicted positive by overexpression of hnRNP A2/B1 (Table 5), 37 (69%) developed lung cancer, whereas of the 40 predicted negative, only 8 (20%) developed lung cancer (relative risk, 3.4; sensitivity, 82%; specificity, 65%) for an overall accuracy of 73%. These data indicate that hnRNP A2/B1 overexpression increased by roughly 3-fold (from 22 to 72%) the sensitivity of routine (Papanicolaou-stained) sputum cell morphology to detect primary lung cancer.

DISCUSSION

Improved understanding of lung cancer biology calls for a fresh look at preclinical detection. We previously reported a promising new early detection approach that labels sputum epithelial cells with two tumor-associated monoclonal antibodies (624H12 and 703D4; Refs. 13, 28, and 32). We reported an 88% diagnostic accuracy of this approach when applied to 62 archived dysplastic (but nondiagnostic) specimens collected 2 years in advance of clinical lung cancer during the JHLP (13). This outcome led directly to the two prospective observational studies now in progress. Results of the present studies, which include the first year of follow-up, are strikingly consistent with our original report. Yet, unlike the original report, these two prospective studies are not limited to an archive of moderately

Table 4 Entry characteristics[a] of 94 subjects at risk of primary lung cancer by outcome group

	Cancer (n = 45)		Control (n = 49)		
	%	Mean (range)	%	Mean (range)	P
Age at enrollment (yr)		62.9 (52–74)		62.7 (52–75)	0.878
Smoking status					0.342
Current	62.2		67.4		
Former	33.3		22.4		
Never	4.4		10.2		
Age at outcome (yr)		62.1 (49–74)	—[b]	—[b]	
Time from sputum to outcome (mo)		15.4 (3.2–36.3)	—[b]	—[b]	
Screening cytoloty					0.006
Normal	57.7		81.6		
Slight	11.1		10.2		
Moderate	8.9		8.2		
Cancer	22.2		0.0		
Cell type of primary					—[b]
Squamous	51.1		—[b]	—[b]	
Adenocarcinoma	4.4		—[b]	—[b]	
Large cell	2.2		—[b]	—[b]	
Other	2.2		—[b]	—[b]	
Missing	40.0		—[b]	—[b]	

[a] Race for all subjects was Chinese, and all were males.
[b] Does not apply.

Table 5 Immunodetection of preclinical primary lung cancer by hnRNP overexpression

Predicted group (test result)	Actual group	
	Cancer	No cancer
Cancer (positive) n = 54	37 (82.2%)	17 (34.7)
No cancer (negative) n = 40	8 (17.8)	32 (65.3)
Total n = 94	45	49

Overall primary lung cancer risk: 56/6285, 0.9%.
Positive predictive value: 37/54, 69%.
Risk among predicted negative: 8/40, 20%.
Relative risk of a positive test: 1480/432, 3.4.
Sensitivity: 82%. Exact 95% binomial confidence interval, 68–92%.
Specificity: 65%. Exact 95% binomial confidence interval, 50–78%.

dysplastic cells. Present results, which do not depend upon morphological preselection, could have potentially wide applicability. This consistency and potential for wide applicability warrant the presentation of these preliminary results.

In these advance reports of two prospective studies, where background cancer risks were 2.2 and 0.9%, respectively, hnRNP A2/B1 overexpression consistently and correctly predicted lung cancer in 67 and 69%, a 35- and 76-fold improvement in positive predictive value. Prospective detection of the lung cancers that developed in the two studies among more than 7000 total persons at risk depended upon computerized measurement of cytoplasmic density of exfoliated airway cells immunostained with monoclonal antibody 703D4. Using criteria developed independently and published previously from JHLP archived specimens, patients at 11 thoracic surgery programs in North America could be classified prior to clinical evidence of SPLC. The robustness of this diagnostic approach is illustrated by a similar level of accuracy among specimens from tin miners at risk of primary lung cancer, collected under more primitive conditions and shipped from China for analysis in Baltimore.

Combining the presentation of preliminary data from both prospective validation studies has provided further insight. In Table 1, the distribution of primary lung cancer cell types illustrates that: (*a*) adenocarcinoma was the most frequently resected primary cell type among those at risk for SPLC; (*b*) the distribution of primary cell types was not different among those who developed a second primary, a recurrence, or remained cancer-free; and (*c*) adenocarcinoma was the most frequently occurring SPLC cell type. By contrast, Table 4 shows that the most frequently occurring primary cell type among YTC miners was squamous cell cancer. As observed in our initial report, the ability of the assay to detect preclinical cancer does not seem to depend directly upon the histology of the subsequent cancer. This observation suggests that hnRNP A2/B1 up-regulation is a feature of preneoplastic cellular transformation, which precedes morphological differentiation.

If hnRNP A2/B1 up-regulation precedes morphological differentiation, is it on the causal pathway of carcinogenesis? Increasing evidence suggests a role for this nucleoprotein early in pulmonary carcinogenesis. It is widely distributed in tissues likely to be undergoing carcinogenesis. A recent study mapping hnRNP A2/B1 overexpression in 16 of 28 primary stage I NSCLC and adjacent uninvolved lung found the presence of hnRNP A2/B1 immunoreactivity in all major histological subtypes (33). Even when bronchi and bronchioli were morphologically normal, hnRNP A2/B1 overexpression was found in one-third of cases, perhaps representing the widespread influence of inhaled carcinogens on the airway (a field effect; Ref. 34). In the alveoli, hnRNP A2/B1 overexpression was most often seen in areas of type II cell hyperplasia.

In addition to clinical evidence, biological data suggest that up-regulation of hnRNP A2/B1 is likely to be found early in carcinogenesis. hnRNP family members have been reported recently as being involved with both growth regulation and cancer (35–41). Levens and co-workers (42, 43) have shown

that hnRNP K not only regulates the transcriptional activity of c-*myc* but also can act as a transcription factor by binding to single-stranded DNA. hnRNP C was shown recently to be a target of a critical apoptosis-mediating protease (44). hnRNP A2 also may play a critical role in regulation of cell death through telomere binding (45–47). Although hnRNP A2 and C differ structurally, they share several common functions (*e.g.*, calmodulin-dependent phosphorylation) and are coexpressed in nonhuman systems (48). hnRNP P2 has been identified as the fusion product resulting from a chromosome translocation t (12, 16), and this fusion product is thought to drive the pathogenesis of certain liposarcomas (34–39). Whether hnRNP A2 overexpression is fundamental to the development of lung cancer or merely associated with cellular transformation remains a focus of study. Understanding this biology may provide additional biomarkers for early detection and monitoring.

Several practical and theoretical issues must be addressed before any improvement in lung cancer prediction can have an impact on lung cancer mortality. Consider the practical concern whether the performance of the hnRNP A2/B1 assay can be generalized to other clinically relevant populations. For discussion, generalizability may be reduced to questions of biased study patient selection and altered disease frequency. Several types of selection bias were avoided by the present designs. All patients were clinically disease free at the start of screening (LCEDWG patients had been surgically staged node negative) so that severity of existing disease did not influence patient participation. In these observational studies, the hnRNP A2/B1 assay results were not revealed to the treating physician and could not influence patient referral, extent of diagnostic studies, end point cancer diagnosis, or treatment. Objective histological end point criteria for SPLC had been already established in similar populations of Lung Cancer Study Group patients. Yet, clearly the frequency of lung cancer in the populations described exceeds that of the general smoking population. It was the higher level of background risk in these populations that gave these small observational studies sufficient power to provide an early indication of hnRNP A2/B1 diagnostic efficacy. We observe that for a comparable annual incidence of lung cancer (LCEDWG, 2.2%; YTC, 0.9%), there follows a comparable increase in lung cancer predictive value after hnRNP A2/B1 testing (to 67 and 69%, respectively). This observation gives confidence that for populations at this level of risk, using sputum cells to monitor hnRNP A2/B1 expression may greatly improve the accuracy of preclinical lung cancer detection. However, conclusive determination of hnRNP A2/B1 predictive value (and demonstration of associated lung cancer mortality reduction of at least 50%) in a general population of current and former smokers with a more typical annual lung cancer incidence of 0.5% must await a prospective intervention trial of more than 10,000 participants similar to the multicenter National Cancer Institute collaboration.

In these studies, hnRNP A2/B1 overexpression has detected preclinical lung cancer with an accuracy similar to the widely used prostate-specific antigen screening test for prostate cancer (49, 50). The significant trends observed in these preliminary results are encouraging, but the data also raise questions that require the completion of the present studies. For example, it is not yet clear whether cigarette smoking will have a significant effect on this assay. If the assay were positive among all current smokers, this may indicate a lack of specificity for detection of preclinical cancer. In Table 2, we observed that cigarette smoking shows a significant association with staining intensity among the SPLC participants, but the larger primary lung cancer study among YTC miners shows a nonsignificant trend of absorbance across smoking status. If cigarette smoking (or other factors) were found to produce a significant modulation of the assay, appropriate adjustment of the discriminant function would be required. Further improvements in specificity also may come from longer follow-up, allowing clinical detection of cases now called falsely positive. Sensitivity may be enhanced by the availability of multiple serial samplings of the exfoliated cells of the airway.

In this report, up-regulation of hnRNP A2/B1 indicated at least a 67% probability of a person's developing lung cancer within 1 year. In the future, additional markers may further improve the accuracy of sputum-based early lung cancer detection (14, 15, 51). Recent developments in diagnostic technology suggest that the ability to do multiple biomarker assays with small clinical specimens at reasonable cost will become routine (52). This advance report is presented now to encourage planning for the large intervention trials needed to determine optimal diagnostic strategies (*e.g.*, localization by fluorescent endoscopy (53), segmental washings/brushings (54), or positron emission tomography scanning). Likewise, planning should be encouraged for evaluation trials of conventional management (*i.e.*, surgery or radiation) compared to newer systemic and airway-delivery chemopreventive approaches (55–58) guided, perhaps, by the intermediate end point of biomarker modulation.

In conclusion, hnRNP A2/B1 is the first of several molecular targets identified as potentially useful lung cancer biomarkers through expression in archived, preneoplastic sputum cells and subsequent tumor. Replicated but preliminary prospective observations support the promise of hnRNP A2/B1 as a lung cancer diagnostic. Additional biomarkers could be evaluated in a similar fashion, permitting the evolution of panels of markers that refine the precision of lung cancer early detection. With progress in lung cancer chemoprevention, these diagnostic developments could be fundamental to a new and more effective range of early lung cancer management options that we now recognize to be essential to supplement traditional prevention efforts.

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